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# Capillary zone electrophoresis–mass spectrometry as a tool in the stability research of the luteinising hormone-releasing hormone analogue goserelin

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**Abstract**

A capillary zone electrophoresis (CZE)–MS system for peptide analysis was developed, characterised and applied in the stability research of goserelin, a luteinising hormone-releasing hormone analogue. 10% acetic acid was used as running solution. An important aspect of this CZE–MS system is its relative insensitivity to injection volume size and ions in sample solutions. As a result of the low pH (2.3) of the running solution, the endoosmotic flow was minimal, resulting in mainly electrophoretic movement and, therefore, relatively high resolution. Degraded samples of goserelin at pH 5 and 9 were subjected to analysis with this CZE–MS system. Information was obtained about the degradation of the C-terminal semi-carbazide group. A major difference between CZE and LC is that goserelin epimers are not separated in the CZE system, whereas they are in the RP-HPLC system. © 1997 Elsevier Science B.V.

*Keywords:* Capillary electrophoresis–mass spectrometry; Stability studies; Goserelin; Peptides

## 1. Introduction

The increasing use of peptides, proteins and peptidomimetics in the pharmaceutical field demands more insight into their (chemical) degradation. These substances are often administered by parenteral injection. Aqueous parenteral solutions are subject to all kinds of degradation, such as hydrolysis, oxidation and racemisation.

The stability in aqueous solution of luteinising hormone-releasing hormone (LH-RH) analogues, peptides of 9–10 amino acid residues, is an important focus of our department's current research. Identification of degradation products is carried out with techniques such as liquid chromatography–mass spectrometry (LC–MS) and (chiral) amino acid analysis.

Because of the complexity of the degradation of the LH-RH analogue goserelin, a separation technique other than LC was desirable, hence capillary zone electrophoresis (CZE) was selected. CZE can be very useful in peptide research, since it provides information about the charge-to-mass ratio of substances in solution. However, it has some practical

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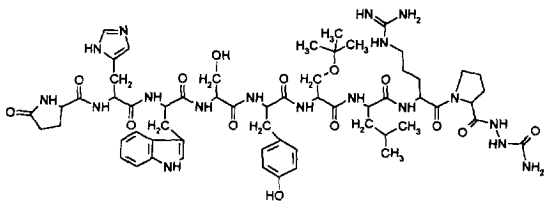

Fig. 1. Chemical structure of goserelin. The $Ser^6$ residue is in the D configuration.

disadvantages. Firstly, resolution in CZE of substances with similar charge-to-mass ratios but different structures is limited [1]. Secondly, the sample capacity of CZE systems is small and, consequently, the sensitivity is relatively low. Finally, ions present in samples can interfere, making analysis unreliable.

If CZE is coupled to a mass detector the limited resolution can be compensated for by the selectivity of the mass detector and sensitivity may be improved. Moreover, substances can be characterised by their mass/charge ($m/z$) values and identified.

Some research has been performed on CZE–MS of peptides [2–5]. Model compounds dissolved, in relatively high concentrations, in pure water are normally analysed, but in general the influence of ionic strength of the sample is not evaluated. Most CZE systems cannot be used when considerable amounts of ions are present in samples, because these ions change strongly the peak efficiency and resolution. This was demonstrated, for example, by Engelhardt and Cunat-Walter [6].

Our goal is to design a robust CZE system, that can be coupled to a mass spectrometer, is insensitive to ions in samples and allows large volumes to be injected (>100 nl). This CZE–MS system is meant to be used in the stability research of goserelin, a nonapeptide with a structure as shown in Fig. 1, in order to identify its degradation products at pH 5 and 9.

## 2. Experimental

### 2.1. Chemicals

Goserelin acetate was obtained from Zeneca Farma (Ridderkerk, Netherlands). All other chemicals were of analytical grade, and water purified by reversed osmosis was applied throughout the study.

### 2.2. Peptide test mixture

A peptide standard mixture, containing angiotensin II, Gly–Tyr, Val–Tyr–Val, leucine-enkephalin and methionine-enkephalin was obtained from Sigma (St. Louis, MO, USA). The Gly–Tyr concentration was set at 10 ppm, all other peptides at 20 ppm. Samples with sodium chloride concentrations of 0, 5, 10, 20, 40 and 100 m$M$ were prepared.

### 2.3. Degraded goserelin samples

Degradation mixtures of goserelin were prepared by heating drug solutions at various pH in a water bath of 70°C. The pH 5 mixture consisted of 580 μg/ml goserelin acetate in 25 m$M$ acetate buffer, degraded over a period of 18 days. The pH 9 mixture contained 1000 μg/ml goserelin and 25 m$M$ borate buffer, degraded over 2 days.

### 2.4. Capillary zone electrophoresis–UV detection

All CZE analysis was performed with a Lauerlabs Prince CE instrument (Separations, Hendrik Ido Ambacht, Netherlands) in a 75 μm I.D. uncoated silica capillary (Bester, Amstelveen, Netherlands), previously rinsed with running solution. Total length of the capillary was 80 cm, with a detection cell at 60 cm from the injector. The voltage over the capillary was set at 30 kV. Detection was performed with an Applied Biosystems 785 A programmable absorbance detector (Separations) operating at 214 nm. Data were acquired with Gynkosoft software version 4.12 (Separations). Two running solutions were used: (1) 20 m$M$ ammonium acetate–40 m$M$ acetic acid in water (pH 4.4), (2) glacial acetic acid–water (10:90, w/w) (pH 2.3).

### 2.5. Capillary zone electrophoresis–mass spectrometry

The CZE system described in Section 2.4 with running solution 2 was coupled to a VG platform II ionspray mass spectrometer equipped with a CE–MS probe (Fisons, Altrincham, UK). The tip of the probe

was earthed. The silica capillary was extended to 120 cm and during injection the nitrogen valve of the MS was closed to prevent pressure disturbances. The sheath liquid consisted of 0.5% (w/w) acetic acid in a mixture of methanol–water (50:50, w/w) delivered to the probe with an LC-10 AD pump (Shimadzu, Tokyo, Japan) at 10 μl/min. The LC-10 AD is specially designed for microflows (<100 μl/min), but to diminish any remaining fluctuations, a T-piece and a 20 cm silica capillary 50 μm I.D. was used to connect the pump with the probe, ensuring an operating pressure of 15 bar. The free end of the T-piece was provided with a 1 mm I.D. metal capillary with a volume of 2 ml, closed at the end. The MS system was operated in the positive ion mode and the scan range was set from 200 to 1000 *m/z* at 500 *m/z* per second. Instrument control and data-acquisition was achieved using Masslynx software version 2.1 (Fisons).

### 2.6. Liquid chromatography–mass spectrometry

The liquid chromatograph consisted of a Waters Model 510 pump, a Waters UK6 injector (both from Waters Associates, Milford, MA, USA). The column used was a Superspher 100 RP-18 (5 μm) 119×2 mm I.D. (Merck, Darmstadt, Germany). The mobile phase consisted of 23% (w/w) acetonitrile in water with 0.1% (v/v) trifluoroacetic acid, the flow was set at 0.1 ml/min and the injection volume was 20 μl. This LC system was directly coupled to a VG platform II mass spectrometer (Fisons), operated in the positive ion mode. The scanned mass range was 100–1500 *m/z* with a scan time of 3 s. Instrumental control and data acquisition were done with MassLynx version 2.1 (Fisons).

## 3. Results and discussion

### 3.1. Development of the CZE system

CE was performed in uncoated 75 μm I.D. silica capillaries. The 75 μm diameter represents an acceptable compromise between volume loadability and sensitivity to hydrodynamic flow when coupled to a mass spectrometer. From experiments with LH-RH analogues, both in our own laboratory as well as from the literature [1], it was found that resolution of these analogues in uncoated silica capillaries was optimal at pH<5. At this low pH, the positive charges of LH-RH analogues located at arginine and histidine residues are responsible for electrophoretic mobility, and at the same time the non-separating electroosmotic flow is reduced due to protonation of silanol moieties at the inner surface of the capillary. Acetate buffers pH 4–5 give reasonable analysis times (<20 min), low currents and good resolution.

Running solution 1 is an example of a buffer used in the literature for CZE–MS. It is tested for compatibility with ions in samples using solutions with mixtures of Lys-bradykinin, angiotensin II and sodium chloride (Fig. 2). The changes in these electropherograms caused by ions are typical for most CZE buffers: if the ionic strength of the sample is equal to or exceeds the ionic strength of the running solution, resolution is diminished. The possibility of increasing the ionic strength of the running solution is limited, because of a higher current accompanied by extra Joule heating. Another in-

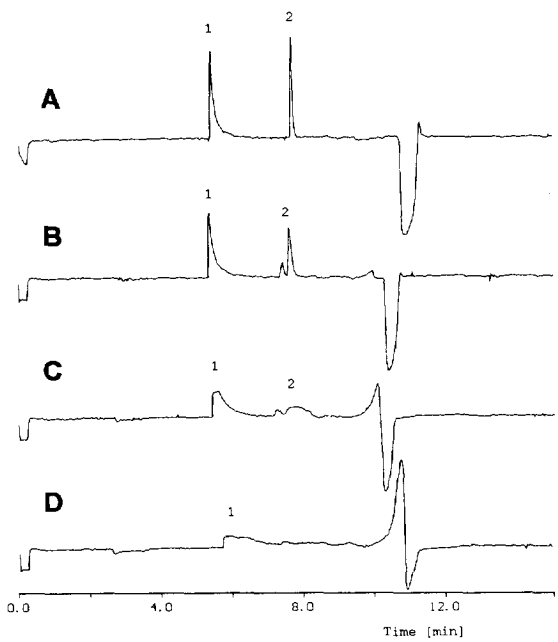


Fig. 2. CZE of 80 ppm Lys-bradykinin and 20 ppm angiotensin II using running solution 1. Peaks are numbered as follows: (1) Lys-bradykinin and (2) angiotensin II. NaCl concentrations in the samples are 0, 20, 50 and 100 m*M* for electropherograms A, B, C and D, respectively. Injection volume: 77 nl (17 mm plug length).

fluence of ions on the CZE system in Fig. 2 is the migration of two peaks of one substance, angiotensin II. This is unacceptable and it can be concluded that running solution 1 is not suitable for our purposes.

When coupling a CZE system to an electrospray mass spectrometer, non-volatile salts in the running buffer must be minimised or even excluded. Acetates should preferably be absent in the running solution. Therefore, experiments were performed with acetic acid only at concentrations of 0.01–0.4 *M* (pH 3.5–2.5). It was observed that the 0.4 *M* solution did not suffer much from ions in the sample, which is remarkable. Also, acceptable separation efficiencies were found of about 40 000 theoretical plates. This might seem low for CZE, but is explained in the equation for calculation of the number of theoretical plates (Eq. (1)) [7]:

$$N = \frac{(\mu_{eo} + \mu_{ep})V}{2D} \quad (1)$$

in which $N$ is the number of theoretical plates, $\mu_{eo}$ represents electroosmotic mobility, $\mu_{ep}$ electrophoretic mobility, $V$ is the voltage and $D$ the diffusion coefficient. The separation efficiency is linearly dependent on the electroosmotic mobility, which is deliberately kept low in this system. Consequently, efficiencies are relatively low but resolution is high, which is required for an adequate separation technique. However, the above described correlation of the separation efficiency and the electroosmotic mobility is only valid when the analytes do not strongly interact with the wall of the uncoated silica capillary.

An even higher acetic acid concentration of 10% (approx. 1.7 *M*) was used by Lu et al. [2], giving good peak shapes and resolution for histidine, tryptophan, phenylalanine, aspartic acid and the dipeptide glutathione. Experiments in our laboratory showed that this high acetic acid concentration gives excellent results with respect to robustness, resolution and conductivity. These are the reasons why 10% acetic acid (running solution 2) is selected for further experiments.

### 3.2. Testing of the CZE system

The characteristics of the CZE system with running solution 2 were tested with a peptide test mixture, containing angiotensin II, Gly–Tyr, Val–Tyr–Val, Leu-enkephalin and Met-enkephalin. Samples of the peptide mixture with an ionic strength ranging from 0 to 0.1 were injected in amounts of 40 to 300 nl. In this way, the resolution, the separation efficiency and influence of ions in samples and injection volumes on resolution and separation efficiency could be measured.

All peptides are baseline separated except the two enkephalin analogues and peak shapes are relatively good, indicating no strong interaction of the peptides with the uncoated silica. The effect of the plug length and ionic strength on the resolution is small, whereas migration times change. In Fig. 3 the robustness of this CZE system is demonstrated by a combination of a large injection volume and a high ionic strength. The resolution of Met- and Leu-enkephalin is partly successful in 100 m*M* NaCl contrary to water. It can be concluded that the

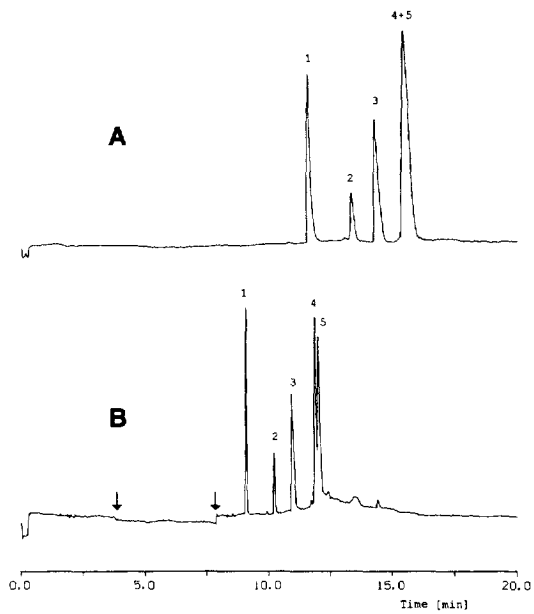


Fig. 3. CZE of peptide test mixture in (A) water and (B) 100 m*M* NaCl using running solution 2. Peaks are numbered as follows: (1) angiotensin II, (2) Gly–Tyr, (3) Val–Tyr–Val, (4) Leu-enkephalin and (5) Met-enkephalin. Injection volume: 306 nl (70 mm plug length). The arrows in B indicate the position of the plug with high $Na^+$ concentration.

influence of ions in samples is mainly limited to changes in migration times.

Fig. 3 suggests that when running solution 2 is used, some kind of stacking occurs from injected plugs with high ionic strength. The mechanism of this stacking has to be different from normal stacking which is generally known to occur in saltless samples.

An indication for the mechanism can be seen in Fig. 3B. Between 4 and 8 min a (reproducible) lowering of the baseline is observed. The length of this zone is linearly related to the plug volume and is not observed when saltless samples are injected (Fig. 3A). The lowering of the baseline was examined with CZE–MS. In every mass scan a background ion with $m/z$ ratio of 214 is present, the composition of which is unknown. However, in the baseline dip the intensity of this ion is relatively small and an extra peak occurs at $m/z$ 236. The mass difference of 22 indicates the substitution of $Na^+$ for $H^+$ in the 214 cluster. This substitution only takes place in the presence of large amounts of $Na^+$ ions and it can be concluded that the lowering of the baseline is caused by passage of $Na^+$ ions through the detector. It seems that ions from the plug first undergo electrophoresis due to their high charge-to-mass ratio, leaving a water plug containing the analyte at the beginning of the capillary. Normal stacking can now take place from this plug, because the applied voltage stands across this plug. When running solutions with ions are used, these ions might replace the ions in the sample plug and this will interfere with possible stacking.

A remarkable phenomenon is that peaks are sharper and resolution is better in Fig. 3B than in Fig. 3A. Apparently, the stacking is more efficient from a sample plug with high ionic strength than from a water plug. Obviously, with samples containing sodium chloride, the analyte plug is preceded by the $Na^+$ zone with a low electric field strength during the stacking process. Analyte ions migrating faster than average, enter the low field strength $Na^+$ zone and will migrate slower. This would reduce peak broadening.

The final test of the CZE–MS system with 10% acetic acid was performed using solutions of goserelin and its analogues gonadorelin, buserelin and triptorelin. All LH-RH analogues are measured as $[M+2H]^{2+}$, due to the basic histidine and arginine residues. The detection limit is 5 ppm or lower and peak shape is excellent.

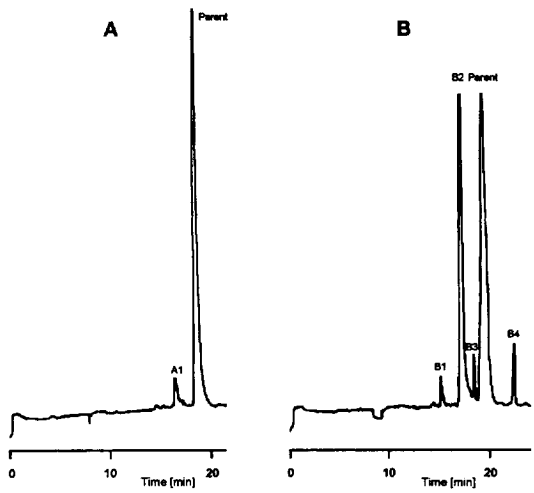


Fig. 4. CZE–UV electropherograms with running solution 2 of goserelin, partly degraded at (A) pH 9 and (B) pH 5. Injection volume: 77 nl (17 mm plug length).

### 3.3. CZE–MS characterisation of the degradation products of goserelin at pH 5 and 9

The CZE–UV electropherograms of two degraded goserelin samples at pH 5 and 9 are shown in Fig. 4. Two peaks are visible at pH 9, and 5 at pH 5.

The CZE–MS results of the sample degraded at pH 9 are summarised in Table 1. Apart from A1, two other products were found, A2 and A3, which coelute with the parent. At pH 2.3, the pH of running solution 2, goserelin has two positively charged

Table 1
CZE–MS characterisation of degraded goserelin at pH 9

| Migration time (min) | $[M+2H]^{2+}$ ($m/z$) | Intensity $[M+2H]^{2+}$ | Name |
|---|---|---|---|
| 18.6 | 614.0 | $5.9 \cdot 10^7$ | Product A1 |
| 19.9 | 626.4 | $3.6 \cdot 10^7$ | Product A2 |
| 20.1 | 635.3 | $3.5 \cdot 10^8$ | Parent |
| 20.2 | 606.9 | $2.1 \cdot 10^7$ | Product A3 |

residues, arginine and histidine. The mass loss of 43 suggests that product A1 is the result of hydrolysis of the C-terminal semicarbazide group leading to acylhydrazin (Fig. 5). From the literature [8] it is known, that free semicarbazide possesses a basic function with a $pK_a$ of approximately 4. This basic function is probably located at the β nitrogen of the hydrazin group, which is also present in the proposed acylhydrazin structure for degradation product A1. A1 is therefore likely to posses three basic functions, which explains the higher mobility at pH 2.3.

Product A2 might be an intermediate in the semicarbazide degradation. From the CZE–MS results it can be derived that there is no charge mutation. The mass difference of −18 suggests loss of a water molecule. However, possible structures for products resulting from water elimination from the semicarbazide group refer to very reactive sub-

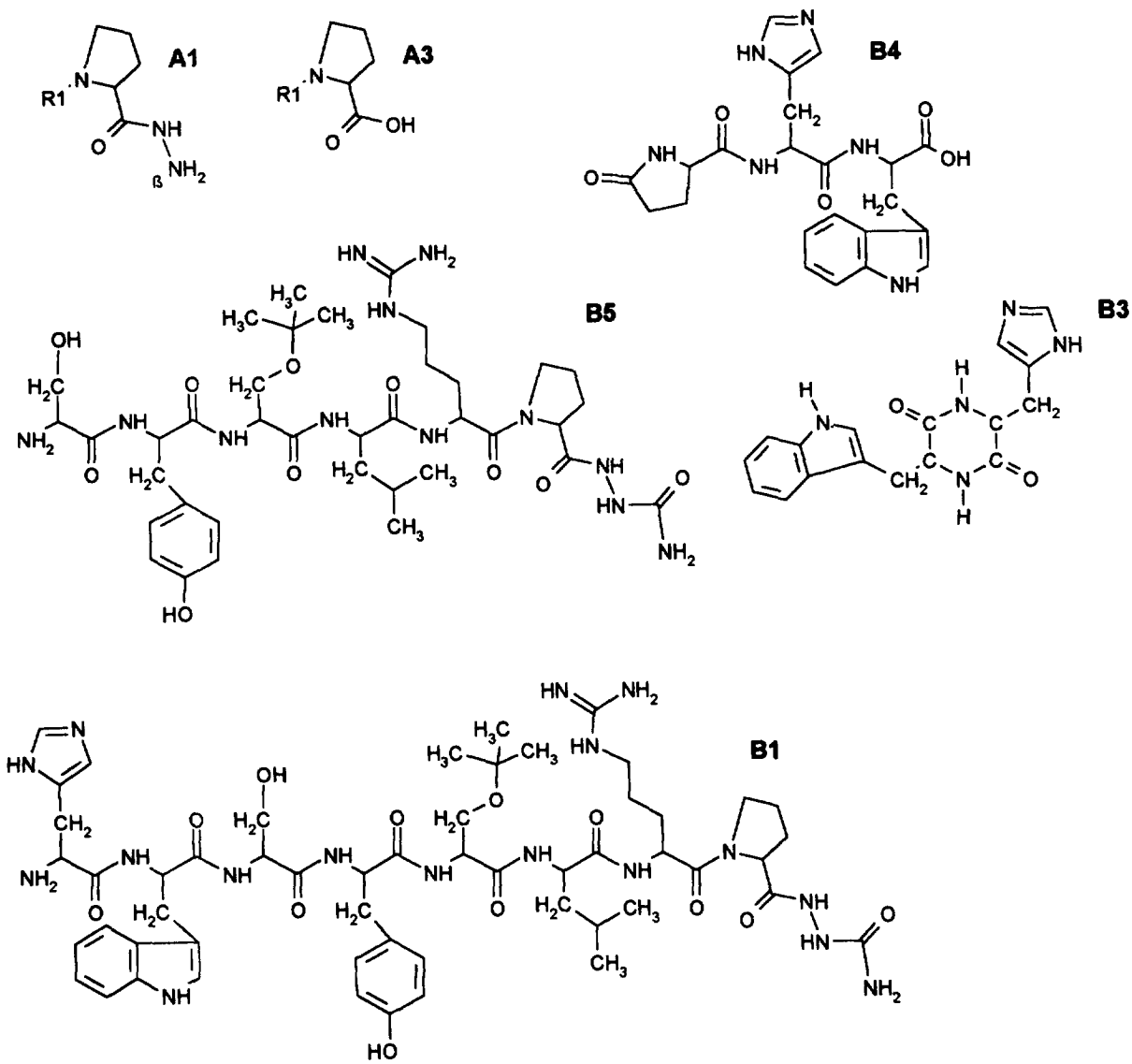


Fig. 5. Chemical structures of products A1 (=B2), A2 (=B6), B4, B5, B3 and B1.

stances, which are not likely to exist in aqueous solution. Future MS–MS research should cast more light on this matter.

Product A3 possibly represents the elimination of the semicarbazide/hydrazine group to the free carboxylic acid (Fig. 5), which is almost fully protonated at pH 2.3. The mobility is similar to goserelin, because size and charge are (approximately) equal. Since $m/z$ ratio 607 is also present as a fragment in the parent mass spectrum, this product can only be discovered by superponation of the reconstructed ion chromatogram (RIC) of $m/z$ 635.5 on the RIC of $m/z$ 607.

At pH 5 more products are detected (Fig. 4 B), and again CZE–MS reveals the identity of two more compounds (Table 2). In Fig. 6 the RICs of all products can be found. Products B2 and B6, are identical to, respectively, products A1 and A3, which probably originate form degradation of the semicarbazide moiety. Products B4+5 result from hydrolysis of the peptide backbone. It is likely that B4 is a tripeptide from the N-terminal site of goserelin (Fig. 5), while B5 is a hexapeptide of the C-terminal site (Fig. 5). The tripeptide can also react to B3, a diketopiperazine consisting of histidine and tryptophan (Fig. 5), as is frequently observed in di- and tripeptides [9]. The peptide bond cleavage followed by diketopiperazine formation was also found for a number of other LH-RH analogues [10–12].

Considering the mass difference of −111, product B1 might be des-pyroglutamyl goserelin (Fig. 5). In this case, B1 would have an extra positive charge due to the N-terminal amino group, which would explain the higher mobility compared to goserelin. The presence of the bulky pyroglutamyl group is responsible for the considerably lower mobility of B2 compared to B1.

### 3.4. Comparison of CZE–MS with 10% acetic acid and LC–MS

The two degraded goserelin samples were also characterised with LC–MS. Results of the sample at pH 9 are shown in Fig. 7. The most striking difference between LC–MS and CZE–MS at pH 9 is that pairs of products with identical $m/z$ ratios have been found with LC–MS, indicating epimerisation, whereas in CZE–MS, $m/z$ ratios are observed only once. Obviously, the resolution of diastereomers in CZE is inferior to RP-HPLC, due to their identical charge-to-mass ratio. However, with CZE–MS products A3 and B1 could be found, which were not visible with LC–MS. Another difference is the large variation in retention times when using LC–MS, making it more difficult to find all degradation products.

The conclusion that LC–MS should be used as standard characterisation technique and that CZE–MS is useful as a complementary technique, especially in the screening for degradation products is justified.

## 4. Conclusions

CZE(–MS) with 10% acetic acid as running solution represents an extremely robust method with respect to ions in samples and injection volume. Most published CZE systems are only tested with saltless samples, which generally means that res-

Table 2
CZE–MS characterisation of degraded goserelin at pH 5

| Migration time (min) | $[M+2H]^{2+}$ ($m/z$) | Intensity $[M+2H]^{2+}$ | $[M+H]^{+}$ ($m/z$) | Intensity $[M+H]^{+}$ | Name |
|---|---|---|---|---|---|
| 19.1 | 579.9 | $7.2\cdot10^{6}$ | | | Product B1 |
| 21.3 | 614.0 | $7.4\cdot10^{7}$ | | | Product B2 |
| 21.7 | 418.1 | $5.6\cdot10^{6}$ | 835.7 | $1.2\cdot10^{6}$ | Product B5 |
| 22.9 | | | 324.2 | $5.7\cdot10^{6}$ | Product B3 |
| 23.9 | 635.5 | $1.3\cdot10^{8}$ | | | Parent |
| 24.3 | 606.9 | $1.1\cdot10^{7}$ | | | Product B6 |
| 27.2 | | | 453.2 | $6.5\cdot10^{6}$ | Product B4 |

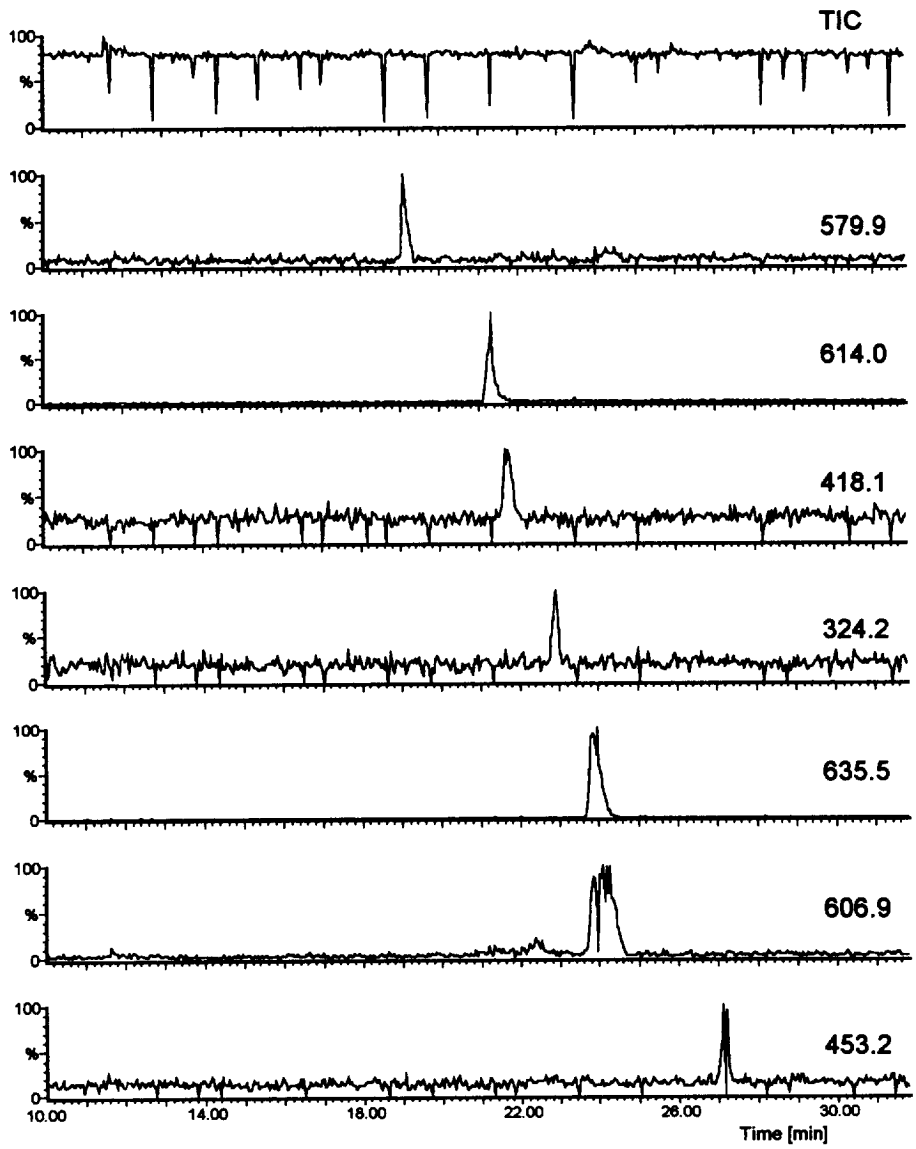


Fig. 6. Total ion electropherogram (TIC) and reconstructed ion electropherograms (RIC) of goserelin degraded at pH 5. In each RIC, the $m/z$ ratio is depicted on the righthand side. Injection volume: 350 nl (80 mm plug length).

olution is diminished when using "daily live" solutions.

CZE–MS with 10% acetic acid is a useful technique in stability research in addition to LC–MS for characterising degradation products of oligopeptides. Other CZE–MS system are not, due to poor resolution and insufficient robustness. The main advantage is that substances elute in a relatively small time window, so the screening for degradation products is faster than with LC–MS. The main disadvantage, compared to LC–MS, is that diastereomers cannot be separated, which was clearly demonstrated with the degradation of goserelin at pH 9. Epimerisation reactions can therefore not be studied with CZE–MS, but the use of a micellar system might overcome this problem.

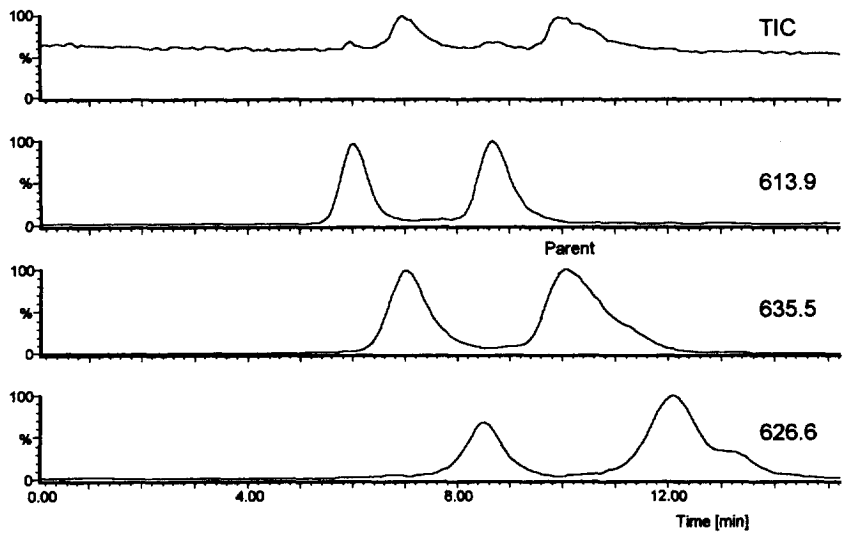


Fig. 7. LC–MS TIC and RICs of goserelin degraded at pH 9. The selected $m/z$ ratios are displayed in the figure.

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## References

[1] P.H. Corran, N. Sutcliffe, J. Chromatogr. 636 (1993) 87.
[2] W. Lu, G. Yang, R.B. Cole, Electrophoresis 16 (1995) 487.
[3] J. Cai, J. Henion, J. Chromatogr. A 703 (1995) 667.
[4] J.H. Wahl, D.R. Goodlett, H.R. Udseth, R.D. Smith, Electrophoresis 14 (1993) 448.
[5] A.J. Tomlinson, S. Naylor, J. Liq. Chromatogr. 18 (1995) 3591.
[6] H. Engelhardt, M.A. Cunat-Walter, J. Chromatogr. A 717 (1995) 15.
[7] R.A. Wallingford, A.G. Ewing, Adv. Chromatogr. 29 (1989) 1.
[8] Merck Index, Merck and Co., Rahway, NJ, 9th ed., 1976, p. 1093.
[9] S. Steinberg, J.L. Bada, Science 213 (1981) 544.
[10] M.G. Motto, P.F. Hamburg, D.A. Graden, C.J. Shaw, M.L. Cotter, J. Pharm. Sci. 80 (1991) 419.
[11] R.G. Strickley, M. Brandl, K.W. Chan, K. Straub, L. Gu, Pharm. Res. 7 (1990) 530.
[12] A.R. Oyler, R.E. Naldi, J.R. Lloyd, D.A. Graden, C.J. Shaw, M.L. Cotter, J. Pharm. Sci. 80 (1991) 271.